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Der Präsident des Europäischen Patentamts;
im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Recombinant infectious non-segmented negative strand RNA virus

The present invention is concerned with a genetically manipulated infectious replicating rabies virus mutant and a process for the preparation of such a mutant.

Rabies virus (RV) is an example of a non-segmented negative-stranded RNA virus of the Rhabdoviridae family. Other species belonging to this family are vesicular stomatitis virus (VSV), infectious hematopoietic necrosis virus (IHNV) viral haemorrhagic septicaemia virus (VHS, Egtved virus), bovine ephemeral fever virus (BEFV), and sonchus yellow net virus (SYNV).

Beside the family of Rhabdoviridae also viruses belonging to the Paramyxoviridae (e.g. sendai virus (SV), para-influenza virus (PIV) type 2 and 3, Newcastle disease virus (NDV), mumps virus (MUV), measles virus (MEV) and canine distemper virus (CDV)) and Filoviridae (e.g. human respiratory syncytial virus (HRSV) and several viruses not assigned to a family (e.g. Borna disease virus; BDV) have a non-segmented negative-stranded RNA genome.

RV can infect all warm-blooded animals, and in nearly all instances after establishment of symptoms the infection ends in death. Dog rabies is still important in many parts of the world: infected dogs cause most of the estimated 75,000 human rabies cases that occur each year world-wide. In many countries of Europe, and in the United States and Canada, wildlife rabies has been increasing in importance.

The clinical features of rabies are similar in most species, but there is great variation between individuals. Following the bite of a rabid animal the incubation period is usually between 14 and 90 days, but may be considerably longer, and incubation periods of over a year have been documented. Two clinical forms of the disease are recognized furious and dumb or paralytic. In the furious form, the animal becomes restless, nervous, aggressive, and often dangerous as it loses all fear of humans and bites at anything that gains its attention. The animal often cannot swallow, giving rise to the synonym for the disease, "hydrophobia". There is often excessive salivation, exaggerated responses to light and sound, and hyperesthesia. As the encephalitis progresses, fury gives way to paralysis, and the animal manifests the same clinical features as seen throughout in the dumb form of the disease. Terminally, there are often convulsive seizures, coma, and respiratory arrest, with death occurring 2-7 days after the onset of clinical signs.

Rabies virus enters the body in the bite or occasionally the scratch of a rabid animal, or when virus-loaded saliva from a rabid animal enters an open wound. Viral replication in the bite site, in muscle, is followed by invasion of peripheral nerve endings and central movement of viral genome in the cytoplasm of axons to the central nervous system. Viral entry into the spinal cord and then the brain (particularly the limbic system) is associated with clinical signs of neuronal dysfunction. Usually, at about the same time that central nervous system infection causes fury, virions are also shed from the apical end of mucus-secreting cells in the salivary glands and are delivered in high concentrations into saliva.

Throughout the course of rabies, host inflammatory and specific immune responses are only minimally stimulated; the most likely reasons for this are because the infection is non-cytopathic in muscle and in nerve cells and because the infection is largely concentrated in the immunologically sequestered environment of the nervous system.

RV virions like all Rhabdoviruses are composed of two major structural components: a nucleocapsid or ribonucleoprotein (RNP) core and an envelope in the form of a bilayer membrane surrounding the RNP core. The infectious component of all Rhabdoviruses is the RNP core. The genomic RNA is of negative sense and thus cannot serve as a messenger but requires its own endogenous RNA polymerase for transcription of mRNA. The RNA genome is encapsidated by the nucleocapsid (N) protein in combination with two minor proteins, i.e. RNA-dependent RNA polymerase (L) and phosphoprotein (P) to form the RNP core. The membrane component contains two proteins: an trans-membrane glycoprotein (G) and a matrix (M) protein located at the inner side of the membrane. The G-protein is responsible for cell

attachment and membrane fusion in RV, and additionally is the main target for the host immune system.

During transcription, the genome directs the sequential synthesis of a short leader RNA and five monocistronic, capped and polyadenylated mRNAs. During replication, the conditional transcription stop and start signals between the cistrons are ignored by the viral polymerase. For both the transcriptase and the replicase reaction the presence of the N-protein complexed with the RNA genome as well as the L- and P-proteins are required. The gene order on the RV genome has been determined and is 3'-leader-N-P-M-G-L-5' as shown in Fig. 1. Each of the mRNAs of RV is translated immediately after transcription. Two events occur sequentially during replication: first the production of an encapsidated complete positive strand RNA complementary to the genome, followed by the production of complete negative stranded RNA which is also encapsidated by the N, L and P proteins. Finally, the newly assembled RNP cores associate with M-protein and G-protein during the assembly and budding process leading to the release of fully formed and infectious RV virions.

The 11.9 kb genomic RV RNA contains five open reading frames (ORFs) coding for the N, P, M, G and L proteins, in addition to the presence of a pseudogene region (ψ) between the G and L genes (Fig. 1).

Current vaccines for non-segmented negative strand RNA viruses comprise chemically inactivated virus vaccines or modified live virus vaccines comprising an attenuated virus strain the pathogenicity of which is decreased by multiple passages in cell culture. Chemically inactivated rabies vaccines are e.g.: Rabivac, Behringwerke (human), HDC, Rhone-Poulenc (human), Bayovac-LT, Bayer (vet), Madivac, Hoechst (vet), Epivax-LT, Pitman-Moore, Rabisin, Rhone-Merieux. For RV examples of such

attenuated viruses are the vaccine strains SAD B19 and ERA. Inactivated vaccines generally induce only a low level of immunity, requiring repeated immunizations. Furthermore, the neutralization inducing antigenic determinants of the pathogens may become altered by the inactivation treatment, decreasing the protective potency of the vaccine.

In general, attenuated live virus vaccines are preferred because they evoke an immune response often based on both humoral and cellular reactions. However, during cell culture passaging uncontrolled mutations may be introduced into the viral genome, resulting in a population of virus particles heterogeneous with regard to virulence and immunizing properties. Over attenuation during passage in cell culture can also be a problem with these vaccines. One must achieve a delicate balance between ensuring that the vaccine is not virulent while making certain that it is still protective. In addition it is well known that such traditional attenuated live virus vaccines can revert to virulence resulting in disease outbreaks in inoculated animals and the possible spread of the pathogen to other animals.

Moreover, a problem with combined live viral vaccines is the mutual influence of the antigenic components resulting in a decrease of the potency of one or more of the constituting components.

Furthermore, with currently administered live attenuated or inactivated RV vaccines it is not possible to determine whether a specific animal is a carrier of RV field virus or whether the animal was vaccinated. Hence, it can be important to be able to discriminate between animals vaccinated with a RV vaccine and those infected with a field virus so as to be able to take appropriate measures to reduce spreading of a virulent field virus. The introduction of for example a serologically identifiable marker can

be achieved by introducing a mutation in a gene encoding a (glyco-) protein of RV which normally give rise to the production of antibodies in an infected host animal.

It is desired to introduce a mutation into the RV RNA genome in a controlled manner such that for example the resulting mutant RV is attenuated or comprises a heterologous nucleic acid sequence encoding epitopes of foreign proteins, e.g. immunological marker proteins or antigens of pathogens. Recombinant DNA techniques are already widely used for this purpose with DNA viruses and positive strand RNA viruses. Examples for recombinant DNA viruses: Aujeszky virus (PRV); Adenoviruses; Vaccinia viruses. Examples for recombinant positive-strand RNA viruses: Alphaviruses (Sindbis V., Semliki forest virus: H.V. Huang, C.M. Rice, C. Xiong, S. Schlesinger (1989) RNA viruses as gene expression vectors. Virus Genes 3, 85-91). Picornaviruses (Polio virus, Hepatitis A-virus, Foot- and mouth-disease virus: J.W. Almond and K.L. Burke (1990) Poliovirus as a vector for the presentation of foreign antigens. Semin. Virol. 1, 11-20). Directed genetic manipulation of RNA virus genomes depends on the ability to produce recombinant RNAs which are accepted as a template by the particular RNA-dependent RNA polymerases. Transcripts generated by many standard DNA-dependent RNA polymerases (e.g. T7 RNA polymerase or cellular RNA polymerase II) and mimicking viral genomes are recognized by the polymerases of many positive stranded RNA viruses. This allowed recovery of infectious viruses or replicons from cDNA transcripts and the application of recombinant DNA technology to manipulate these genomes in a site specific manner. Since RNAs corresponding to the genomes of positive stranded RNA viruses may function as mRNA for translation of the viral polymerases, an infectious

cycle may be initiated by introduction of the genome analogs into a cell. The template of the polymerases of negative stranded RNA viruses, however, exclusively is the RNP complex. Moreover, and in contrast to positive stranded RNA viruses, their genomic or antigenomic RNA may not function as mRNA and thus all viral proteins involved in replication and transcription of artificial RNAs have to be provided in trans.

An appropriate system for encapsidation of genomic RNA analogs of a negative stranded RNA viruses with a segmented genome in order to provide the appropriate template is recently disclosed by Palese, P. et al., (WO 91/03552). RNA transcripts from influenza virus genome segments were encapsidated by purified proteins in vitro which can be used to transfect cells together with a helper virus. However, it was found that this approach was not successful with RV, a virus having a non-segmented genome. Short model genomes of VSV and RV lacking the major part of the RNA genome comprising the genes encoding the viral proteins could be encapsidated and expressed by plasmid encoded proteins (Pattnaik, A.K. et al, Cell 69, 1011-1020, 1992; Conzelmann, K-K. and M. Schnell, J. Virology 68, 713-719, 1994). This approach involved the co-expression of both the genome analogs optionally comprising reporter gene inserts, and particular viral proteins from transfected plasmids in order to produce defective virus particles. Attempts to obtain infectious recombinant negative stranded RNA viruses with a large, non-segmented genome which necessitates manipulation of the entire genomes, have failed until now.

The present invention provides a genetically manipulated infectious replicating rabies virus (RV) mutant, obtainable by recombinant DNA techniques, comprising an insertion and/or deletion in an ORF, pseudogene region or non-coding region of the RV genome.

The insertion and deletion of one or more nucleic acid residues can be introduced in the RV genome by incorporating the appropriate mutations into the corresponding viral ORF, pseudogene region or non-coding region. This alteration is understood to be a change of the genetic information in the RV ORF or pseudogene of a parent RV thereby obtaining the insertion or deletion RV mutant according to the invention.

A mutation, in which one or more nucleotides are replaced by other nucleotides, a socalled substitution replacement is considered to be the result of a combined deletion and insertion action. This kind of mutation is therefore also considered to be included in the wording: deletion and(/or) insertion.

It is clear that any mutation as defined herein comprises an alteration of appropriate RV sequences such that the resulting RV mutant is still infectious and replicating, i.e. the mutant RV is capable to infect susceptible cells and its mutant RNA genome is capable of autonomously replication and transcription, i.e. no co-expression of RV N, P and L proteins is required.

The genomic organisation of different RV strains is identical. The nucleotide sequence and deduced amino acid sequence analysis of the vaccine strain SAD B19 and the virulent strain PV have been determined (Conzelmann et al., Virology 175, 485-499, 1990 and Tordo et al., Nucleic Acids Res. 14, 2671-2683, 1986; Proc. Natl. Acad. Sci USA 83, 3914-3918, 1986; Virology 165, 565-567, 1988). In Conzelmann et al., 1990 (supra) it is determined that the viral genome of the SAD B19 strain comprises 11.928 nucleotides and that the deduced amino acid sequence of the five viral proteins N, P, M, G and L are highly similar to those of the pathogenic PV strain. The location of the respective ORFs, pseudogene region and intergenic non-coding regions in RV have been determined therein: the coding region of the RV N, P, M, G and L genes correspond with positions 71-1423, 1514-2407, 2496-3104, 3317-4891, 5414-11797, respectively. The pseudogene region ( ) maps at position 4961-5359, whereas the intergenic regions separating the five cistrons and which are flanked by non-coding sequences containing transcriptional start and stop/poly-adenylation signals map to positions 1483-1484; 2476-2480; 3285-3289; 5360-5383. Although the numbering and the nucleotide sequence of the ORFs, pseudogene region or non-coding regions of the parent RV strain used herein to introduce a mutation is not necessarily the same as that of the SAD B19 or PV strain, the above-mentioned characterisations of these regions exactly define the localisation thereof on the genome of any RV strain.

A method to obtain an attenuated RV from a virulent parental RV strain is to introduce the insertion and/or deletion in an ORF encoding a viral protein, for example such that the activity of the viral protein for host cell attachment and membrane fusion is modified, e.g. reduced. It is known for RV that changes in the amino acid sequence of the transmembrane glycoprotein G have significant effects on the pathogenicity of the RV. In addition, with regard to attenuation also changes in the matrix (M) protein may influence the conformation of the G protein resulting in an attenuation of the virus. Therefore, mutant RV comprising a deletion or insertion in the ORF encoding the G or M protein are particularly preferred herein.

Alternatively, attenuation of the RV may be obtained by altering the enzyme activity of the RV replicase or transcriptase so that the enzyme is less active, thereby resulting in the production of less infectious virions upon infection of a host animal. As the N, P and L proteins are involved in the RV polymerase activity, RV mutants having an insertion or deletion in the ORF encoding the N, P or L proteins are also part of the invention.

RV deletion and/or insertion mutants according to the invention can also be used to vaccinate a host in order to be able to discriminate (serologically) between a host to which a vaccine comprising said RV mutant is administered and a host infected with a parental RV. In this embodiment of the invention the insert in the RV insertion mutant may encode a heterologous epitope which is capable of eliciting a specific non-RV immune response in an inoculated host, or may encode a protein with enzymatic activity, such as CAT or lacZ (Conzelmann and Schnell, 1994, supra).

A preferred region for the incorporation of such inserts is the RV pseudogene region. As is demonstrated in the Examples insertions and deletions can be made in this region without disrupting essential functions of RV such as those necessary for infection or replication. The RV deletion mutant may lack an epitope of a RV protein against which an immune response is normally raised by the vaccinates, in particular a RV mutant comprising a deletion in the ORF encoding the G protein is suited for this purpose. In the case of a RV insertion mutant the insertion comprises a nucleic acid sequence encoding a serological marker antigen or an epitope thereof.

In a further embodiment of the invention a RV mutant is provided which is capable of expressing one or more different heterologous epitopes or polypeptides of a specific pathogen. Such a mutant can be used to vaccinate animals, both domestic and non-domestic animals, against wildlife rabies and said pathogen.

Vaccination with such a live vector vaccine is preferably followed by replication of the RV mutant within the inoculated host, expressing in vivo the heterologous epitope or polypeptide along with the RV polypeptides. The polypeptides expressed in the inoculated host will then elicit an immune response against both RV and the specific pathogen. If the heterologous polypeptide derived from the specific pathogen can stimulate a protective immune response, then the animal inoculated with the RV mutant according to the invention will be immune to subsequent infection by that pathogen as well as to infection by RV. Thus, a heterologous nucleic acid sequence incorporated into a suitable region of the RV genome may be continuously expressed in vivo, providing a solid, safe and longlasting immunity to the pathogen.

In particular, the present invention provides a RV vector which comprises an insertion of a nucleic acid sequence encoding an epitope or polypeptide of a specific pathogen, wherein the insertion is made in the pseudogene region.

If desired, part or whole of the pseudogene region can be deleted in the RV vector described above.

Preferably nucleic acid sequences encoding an epitope or polypeptide of canine parvovirus, canine coronavirus and classical swine fever virus (CSFV) are contemplated for incorporation into a suitable region of the RV genome.

The possibility to manipulate the non-segmented negative stranded RNA genome of RV on the DNA level by recombinant DNA techniques was not possible until now, because no infectious replicating virus could be generated. However, a process is provided herein which allows the engineering of a mutation into a coding region or non-coding region of the viral genome on the DNA level by means of recombinant DNA techniques followed by the generating of an infectious replicating RV harbouring the mutation in its genome.

This process according to the invention comprises the steps of

a) introducing into cells expressing a RNA polymerase;
   1) one or more DNA molecules encoding the RV N, P and L proteins, and
   2) a DNA molecule comprising the RV cDNA genome and
b) isolating the viruses produced by the cells.

Normally, the cDNA of the rabies virus genome is modified by the incorporation of a mutation in the genome.

The process may however also be used to e.g. purify contaminated RV pools. In that case, the original non-mutated cDNA will be used.

In view of the fact that rescue efficiency of a model mini-genome of RV comprising heterologous inserts with plasmid encoding proteins is extremely low and moreover correlates with insert length (Conzelmann and Schnell, 1994, supra) it could not be expected that initiation of a productive infection from transfected full length genomic RNA could be achieved by co-transfection with plasmids encoding the RV N, P and L proteins. This is the more so as large amounts of positive sense N, P and L specific RNAs are produced from the transfected protein encoding plasmids which were expected to hybridize with simultaneously expressed negative stranded genomic RNA transcripts. Possible hybridization, however, which could affect more than half of the genome was suspected to interfere with the crucial encapsidation step. In addition, translation of N, P and L mRNA might be affected. Indeed it was found that with the standard transfection protocol no infectious viruses could be obtained. However, as demonstrated in the examples the application of an alternative transfection protocol in combination with the use of a RV cDNA genome generating positive stranded antigenomic RNA transcripts, gave rise to a replicating genetically engineered RV.

The above-mentioned process allows the in vitro incorporation of a mutation in the genome of a parental RV by means of recombinant DNA techniques followed by the generation of an infectious replicating RV mutant harbouring said mutation. The mutation includes but is not limited to an insertion, deletion or substitution of nucleic acid residues into an ORF encoding a RV protein, a non-coding region e.g. the pseudogene region, or a transcriptional signal sequence of RV parental genome.

The engineering of a mutation in a non-coding intergenic region may influence the transcription of a specific viral gene such that the transcription of the mRNA and the subsequent translation of the protein, either an envelope protein, such as the M and G protein or a protein involved in polymerase activity, such as the N, P or L protein, is reduced resulting in a virus mutant featuring attenuated characteristics because the mutant's capability of producing (infectious) progeny virus is reduced. In particular the substitution of one or more nucleic acid residues in this intergenic region and/or transcriptional signal sequences can influence efficiency of transcription.

Furthermore, the substitution of one or more nucleic acid residues in a region of the genome of a virulent RV which is involved with virulence, such as the ORF encoding the G protein, by the application of the process described herein is part of the invention.

Such a mutation may result in the exchange of a single amino acid in the G protein of a virulent RV strain resulting in a (partial) loss of pathogenicity, e.g. replacement of Arg (333) with Ile, Glu or Gln, or Leu (132) by Phe, or Trp.

In the process according to the invention the DNA molecule containing the RV genetic information preferably comprises a plasmid provided with appropriate transcription initiator and terminator sequences recognizable by a polymerase co-expressed by the transfected host cells.

A preferred process according to the invention comprises the use of host cells transfected with RV DNA, said cells being able to express bacteriophage T7 DNA-dependent RNA polymerase, expressed for example cytoplasmically from vaccinia virus recombinant. In this case the plasmids containing RV DNA are provided with the T7 promoter and terminator sequences (Conzelmann and Schnell, 1994, supra).

For the preparation of a live vaccine the recombinant RV mutant according to the present invention can be grown on a cell culture derived for example from BHK, or human diploid cells. The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilized. In addition to an immunogenically effective amount of the recombinant RV the vaccine may contain a pharmaceutically acceptable carrier or diluent.

Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol $F^{(R)}$ or Marcol $52^{(R)}$, saponins or vitamin-E solubilisate.

The useful dosage to be administered will vary depending on the type of mammal to be vaccinated, the age, weight and mode of administration.

The dosage may vary between wide ranges: 10^2 to 10^7 pfu/animal would e.g. be suitable doses.

A specific dosage can be for example about 10^6 pfu/animal.

A RV mutant according to the invention can also be used to prepare an inactivated vaccine.

For administration to animals, the RV mutant according to the present invention can be given inter alia orally, intranasally, intradermally, subcutaneously or intramuscularly.

The RV vaccine according to the invention can be administered to dogs but also to the main vectors, i.e. raccoons, skunks and foxes. Furthermore, also vaccination of wild boars with a live RV vector capable of expressing a heterologous gene of a porcine pathogen such as classical swine fever virus, is contemplated.

Example 1

Preparation of infectious replicating RV virions

Construction of full length RV cDNA (Fig. 2).

The cloning of cDNA spanning the entire genome of RV strain SAD B19 was described previously (Conzelmann et al., 1990, supra; GenBank accession number M31046). The numbering of RV nucleotides and amino acids used herein corresponds to that of Conzelmann et al., 1990 (supra). As basis for the assembly of a SAD B19 full length DNA clone the RV mini-genome sequence contained in the transcription plasmid pSDI-1 (Conzelmann and Schnell, 1994, supra) was used (Fig.2). pSDI-1 contains the SAD B19 genomic 3′ and 5′ ends (SAD B19 nucleotides 1-68 and 11760-11928, respectively) inserted between a T7 RNA polymerase promoter and the

hepatitis delta virus (HDV) antigenome ribozyme sequence. In order to generate a plasmid to produce positive stranded SDI-1 transcripts (pSDI-1plus) the RV sequences contained in pSDI-1 were first amplified by PCR using an 11 base primer (5'-ACGCTTAACAA-3') which due to the complementary of RV genome ends corresponds to the 5' termini of both positive and negative sense viral RNAs. After subsequent partial ligation of a synthetic EcoRI/blunt adaptor (T7/3) containing a T7 promoter sequence followed by three G residues (underlined) (5'-AATTCCTGCAGTAATACGACTCACTATAGGG-3') to the amplified RV sequence, the ligation products were cloned in the EcoRI/SmaI sites of pX8dT. This plasmid is a derivative of pBluescriptII (Stratagene) from which a BssHII/ClaI fragment of the multiple cloning site containing the original T7 promoter was deleted. It contains the 84 base HDV antigenomic ribozyme sequence in the SmaI site followed immediately by a T7 transcription terminator sequence cloned in the BamHI site. Constructs that contained a T7 promotor upstream of the plus sense RV sequence were identified by restriction analysis and sequencing. The MunI-Bg1II fragment of pSDI-1 (SAD B19 nucleotides 40-68) was then replaced with a 1 kb MunI/Bg1II cDNA construct assembled in pBluescriptII from three fragments of different SAD B19 cDNA clones (MunI-SphI (SAD B19 nucleotides 40-482 from pZAD1-9); SphI-AatII (4041-4273 from pSAD13), and AatII-Bg1II (11472-11759 from pSAD85)) resulting in pSDI-1170. By insertion of a SphI fragment assembled from the clones pSAD25 and pSAD13 via NcoI (SAD B19 nucleotides 482-4041) and an AatII fragment assembled from clones pSAD 49 and pSAD85 via XhoI (SAD B19 nucleotides 4273-11472) into the unique SphI and AatII sites of pSDI-1170, the final basic full length clone pSAD L16 was completed.

Using the circular plasmid, in vitro transcriptions were performed and the products analyzed on denaturing agarose gels. The presence of RNA transcripts co-migrating with 12 kb RV genomic RNA indicated that full length antigenome RNA is transcribed by T7 polymerase.

Recovery of infectious recombinant RV

The co-transfection of plasmid pSAD L16 and plasmids encoding RV proteins N, P and L was carried out as described in Conzelmann and Schnell, 1994 (supra).

Transfection experiments were carried out as described previously. BHK-21, clone BSR cells were grown overnight in 3.2 cm-diameter dishes in Eagle's medium supplemented with 10% calf serum to 80% confluence, and infected at a m.o.i. of 5 with the recombinant vaccinia virus vTF7-3 (Fours et al., Proc. Natl. Acad. Sci USA 83, 8122-8126, 1986). One hour pontificating cells were washed twice with culture medium lacking calf serum and transfected with a plasmid mixture containing 5 µg pT7T-N, 2.5 µg pT7T-P, and 2.5 µg pT7T-L and with 2 µg of pSAD-L16 plasmid by using the mammalian transfection kit (Stratagene; $CaPO_4$ protocol) according to the suppliers instructions. The precipitate was removed 4 h posttransfection and cells were washed and incubated in Eagle's medium containing 10% calf serum. Possible encapsidation of pSAD-L16 derived T7 RNA polymerase transcripts and the resulting expression of RV proteins from the nucleocapsids was checked by indirect fluorescence. A monoclonal antibody directed against RV G protein, which could only be expressed from the recombinant RV genome, was used to screen the cultures. One day after transfection stained cells were present, demonstrating expression of genes from

the RV genome. However, only single positive cells were observed in a series of 20 transfection experiments. No fluorescent cell foci indicating the presence of infectious virus were obtained in these experiments. In addition, from cell cultures which were inoculated with the entire supernatant from the transfected cells no infectious virus could be recovered two days later. Therefore, in order to isolate a presumed very low number of infectious virus generated in transfected cells, the experimental procedure was modified. For isolation of transfectant viruses cells and supernatants were harvested 2 days post transfection. Cells were suspended in the supernatant by scratching with a rubber policeman. The suspension was submitted to three cycles of freezing and thawing (-70 °C/37 °C, 5 min each). Cellular debris and the excess of vaccinia virus which forms aggregates under these conditions was pelleted by 10 min of centrifugation at 10.000 g in a microfuge. The entire supernatant was used to inoculate a culture dish with a confluent monolayer of cells. After incubation for 2 h, the supernatant was replaced by 2 ml of fresh culture medium. A cythopathogenic effect (cpe) caused by vaccinia virus was observed one to two days post infection. In average only ten plaques were observed after centrifugation at 10.000 g. RV infection of cells, which does not result in detectable cpe was demonstrated two days post infection by direct immunofluorescence staining of the entire monolayer with an anti-N conjugate (Centocore). In two out of 20 experiments fluorescent foci were observed and the respective supernatants contained infectious RV (SAD L16) which was assumed to represent transfectant virus generated from cDNA transcripts.

Half of the supernatants from the cultures in which foci were observed, was used for the second passage after centrifugation at 10.000 g. For further passaging (2 days each) decreasing aliquots of supernatants were used according to the degree of RV infection. To get completely rid of Vaccinia virus, supernatants from cultures approaching infection of all cells (third passage) were centrifuged two times for 10 min at 14.000 g in a microfuge. The final supernatant was then filtered using a sterile MILLEX-VV 0.1 µm filter unit (Millipore Products, Bedford, MA 01730) and then used to produce high titre stocks of recombinant RVs.
The latter transfection and isolation protocol was used in the subsequent Examples.

Example 2

Insertion of an oligonucleotide in the RV pseudogene region

Manipulations of the ψ were carried out in the sub-clone pPsiX8, containing a 2.8 kb XhoI-ScaI fragment of pSAD L16 representing SAD B19 nucleotides 3823 to 6668. The StuI fragments of the modified pPsiX8 plasmids were then isolated and used to replace the corresponding fragment (SAD B19 position 4014 to 6364) of the full length clone pSAD L16 (Fig. 1). Insertion of 4 nucleotides into the ψ and generation of a novel NheI site was achieved by digestion of pPsiX8 with Hind III, fill in of the extensions with Klenow enzyme and religation. The final full length clone pSAD U2 is distinguished from SAD L16 by the duplication of nucleotides 5338 to 5341.

The generation of infectious viruses was demonstrated after transfer of extracts from transfected cells together with supernatant to fresh cells. In each of the series focus formation was observed in one experiment. The transfectant viruses (clones SAD U2-13 and SAD U2-32) were passaged by transfer of supernatants to fresh cells two further times resulting in almost 100% infection of the cells. To demonstrate the insertion in the SAD U2 virus genome, total RNA was isolated from cells infected with SAD U2-13 and reverse transcriptase-PCR (RT-PCR) of the ψ was performed. With the primers G3P and L4M (Fig. 1), which are specific for the G and L genes, respectively, DNA fragments of approximately 730 bp were obtained from the genomes of transfectant viruses SAD U2 and SAD L16 and of standard RV SAD B19. However, subsequent digestion with HindIII was only observed for the PCR DNA obtained from SAD B19 and SAD L16, but not for that from SAD U2. Conversely, only SAD U2 derived DNA was digested with NheI, giving rise to two fragments of approximately 530 and 200 bp, respectively (Fig. 3). Direct RT sequencing of genomic RNA of transfectant virus SAD U2 further confirmed the presence of the expected insertion of 4 residues at the predicted site, while the rest of the determined sequence corresponded to that of the original SAD B19 genome. Thus, it was clear that SAD U2 virus represented a transfectant virus whose genome originated from engineered cDNA.

The introduction of four additional nucleotides close to the end of the RV ψ did not affect viability of the transfectant virus SAD U2, nor did it interfere with correct transcription termination of the G mRNA.

Example 3

Alteration of RV transcription by an insertion or deletion between G and L coding region

By double digest with StyI and HindIII, Klenow fill in and religation, 396 bases (SAD B19 nucleotides 4942 to 5337) were deleted, the final construct was pSAD W9. For the construction of pSAD V*, a 180 bp BgIII-AsuII fragment including the SAD B19 N/P cistron border region was isolated from pSAD13 (Conzelmann et al., 1990, supra). The fragment contained 97 nucleotides of the N coding region, the entire 3' non-coding region and the N/P cistron border consisting of the N transcriptional stop/polyadenylation signal, the intergenic region, and the first 16 nucleotides of the P cistron including the transcriptional start signal. The cDNA fragment was first sub-cloned into the EcoRI site of pBluescript after fill-in of 3' recessive ends with Klenow enzyme (pNigP-180). After excision with HindIII/XbaI from pNigP and blunt end generation the obtained 230 bp fragment which contained the RV insert flanked by 16 and 34 bp of vector derived sequences, respectively, was cloned into the filled-in StyI of pPsiX8. The final full length construct (pSAD V*) thus possessed a 234 bp insertion compared to pSAD L16.

As before, pSAD V* and pSAD W9 were used to transfect twenty culture dishes each. In three cultures transfected with SAD V* and in one with SAD W9, rescue was indicated by subsequent isolation of viable virus. After five successive passages RNA from infected cells and supernatant was isolated and analyzed by RT-PCR using the same primers as in the previous experiments. In comparison to standard SAD B19 virus, an enlarged DNA fragment of approximately 0.9 kb resulted from RNA of cells infected with SAD V* thus showing that additional sequences were present in

the region of this transfectant virus (Fig. 4). In contrast, from RNA of cells infected with SAD W9, a DNA fragment of only 0.3 kb was obtained; this size was expected according to the deletion made in the cDNA genome copy. Sequencing of PCR products confirmed further that the original engineered cDNA sequences were rescued into the genomes of SAD V* and SAD W9 transfectant viruses. Accordingly, neither the presence of additional sequences, including 50 vector derived nucleotides, between the G open reading frame and the ψ nor the deletion of the entire ψ did interfere with the infectivity and propagation of transfectant rabies viruses. The alterations engineered into the genomes of SAD V* and SAD W9 were designed in a way to result in phenotypical changes in the transcription pattern and it was investigated whether this affected the growth characteristics of the respective transfectant viruses. However, propagation in cell culture as well as final titers of infectious SAD V* and SAD W9 viruses were similar to those of standard SAD B19 RV. Three days after infection of cells with an m.o.i. of 0.01, titers of 10^8 focus forming units (ffu) were reached in the supernatants for SAD B19, SAD V* and SAD W9 demonstrating that the RV ψ is not essential for propagation in cell culture.

Using a ψ specific probe, no hybridization was detected with RNA from cells infected with the ψ - deleted SAD W9 virus. While the genomic RNAs of the other viruses and the G mRNAs of SAD B19 and SAD L16 were recognized by this probe, the SAD V* G mRNA did not react. In contrast, a faint band of RNA appeared that corresponded in size to the novel extra ψ-mRNA that was predicted by the presence of the extra P gene transcriptional start signal preceding the SAD V* ψ sequences. In contrast to naturally occurring RV, the

transfectant virus SAD V* represents a RV whose genome is composed of six functional cistrons.

LEGENDS TO FIGURES

Fig. 1:
Organization of the RV pseudogene region (ψ) and construction of recombinant RV genomes (drawn to scale). Numbers indicate the nucleotide positions in the anti-genome sequence of SAD B19. On top, the entire RV genome with its five open reading frames is shown. Mutations were carried out in pPsiX8 containing part of the genome (3823-6668) and reintroduced into the full length clone pSAD L16 by exchange of the StuI fragment (4014-6364). In the detail drawing, coding regions are represented by gray boxes, non-coding sequences as lines. Functional transcriptional signal sequences are indicated by filled bar (stop/polyadenylation) and arrowhead (mRNA transcription start). The nonfunctional signal-like sequence defining the start of the ψ region is shown by the open bar. Arrows indicate the position of oligonucleotide primers G3P and L4M used for RT-PCR analysis of the ψ region. In SAD U2, fill-in of HindIII extensions resulted in insertion of 4 nucleotides and generation of a unique NheI-site. In SAD V*, a cDNA fragment containing the RV N/P cistron border (SAD B19 nucleotides 1323-1502) was inserted into the StyI site; SAD W9 possesses a deletion of the StyI/HindIII fragment.

Fig. 2:
Simplified scheme for the construction of transcription plasmids containing full length RV cDNA. Numbers refer to nucleotide positions of the SAD B19 RV antigenome sequence (Conzelmann et al., 1990). The plasmid pSDI-1plus which served as a basis for reconstruction of full length RV genomic DNA is a counterpart of pSDI-1 (Conzelmann and Schnell, 1994) containing the SDI-1 RV mini-genome that comprises the terminal nucleotides 1-68 and 11760-11928 in opposite direction with respect to T7 RNA polymerase promoter (T7) and hepatitis delta virus antigenomic ribozyme sequence (HDV). The MunI-BglII fragment of pSDI-1plus was replaced with a 1 kb cDNA construct that was assembled from three SAD B19 cDNA clones as indicated. Insertion of a 3.6 kb SphI and a 7.2 kb AatII fragment which were assembled from two cDNA clones each resulted in the final plasmid pSAD L16 containing full length SAD B19 cDNA. Transcription of this plasmid by T7 RNA polymerase should yield positive stranded (antigenomic) RNA possessing three extra non-viral G residues at the 5′ and a precise 3′ end after autolysis of the ribozyme. (T7) T7 promoter; (T7T) T7 transcription terminator; (HDV) HDV antigenomic ribozyme sequence.

Fig. 3:
Demonstration of the genetic tag in the genome of the transfectant virus SAD U2.
Total RNA from cells infected with standard RV SAD B19 (B19) and transfectant viruses SAD L16 (L16) and SAD U2 (U2) was isolated 2 days post infection and used for RT-PCR amplification of the respective ψ regions with primers G3P and L4M. The amplified DNA was separated in a 1% agarose gel directly and after digestion with HindIII and NheI, respectively. A NheI restriction site is present only in DNA derived from SAD U2.M,DNA size marker.

Fig. 4:
PCR analysis of SAD B19 (B19), SAD V* (V*), and SAD W9 (W9) genomes. RT-PCR was performed as described in fig. 3 with primers G3P and L4M. Amplification products were separated in a 1% agarose gel.

CLAIMS

1. A genetically manipulated infectious replicating rabies virus mutant comprising an insertion and/or deletion in an open reading frame, a pseudogene region or an intergenic region of the rabies virus genome.

2. A rabies virus mutant according to claim 1, characterized in that the rabies virus comprises an insertion and/or deletion in an open reading frame.

3. A rabies virus mutant according to claim 2, characterized in that the rabies virus comprises an insertion and/or deletion in the open reading frame encoding the glycoprotein G.

4. A rabies virus mutant according to claim 1, characterized in that the rabies virus comprises an insertion and/or deletion in the pseudogene region.

5. A vaccine for the prevention of rabies virus infection in a mammal, characterized in that the vaccine comprises a rabies virus mutant according to claims 1-4 and a pharmaceutically acceptable carrier or diluent.

6. A process for the preparation of an infectious replicating rabies virus comprising the steps of
   a) introducing into a host cell expressing a RNA polymerase;
      1) one or more DNA molecules encoding the rabies virus N, P and L proteins, and
      2) a DNA molecule comprising the cDNA of the rabies virus genome
      and
   b) isolating the viruses produced by the cells.

7. A process according to claim 6, characterised in that the cDNA of the rabies virus genome is modified by the incorporation of a mutation.

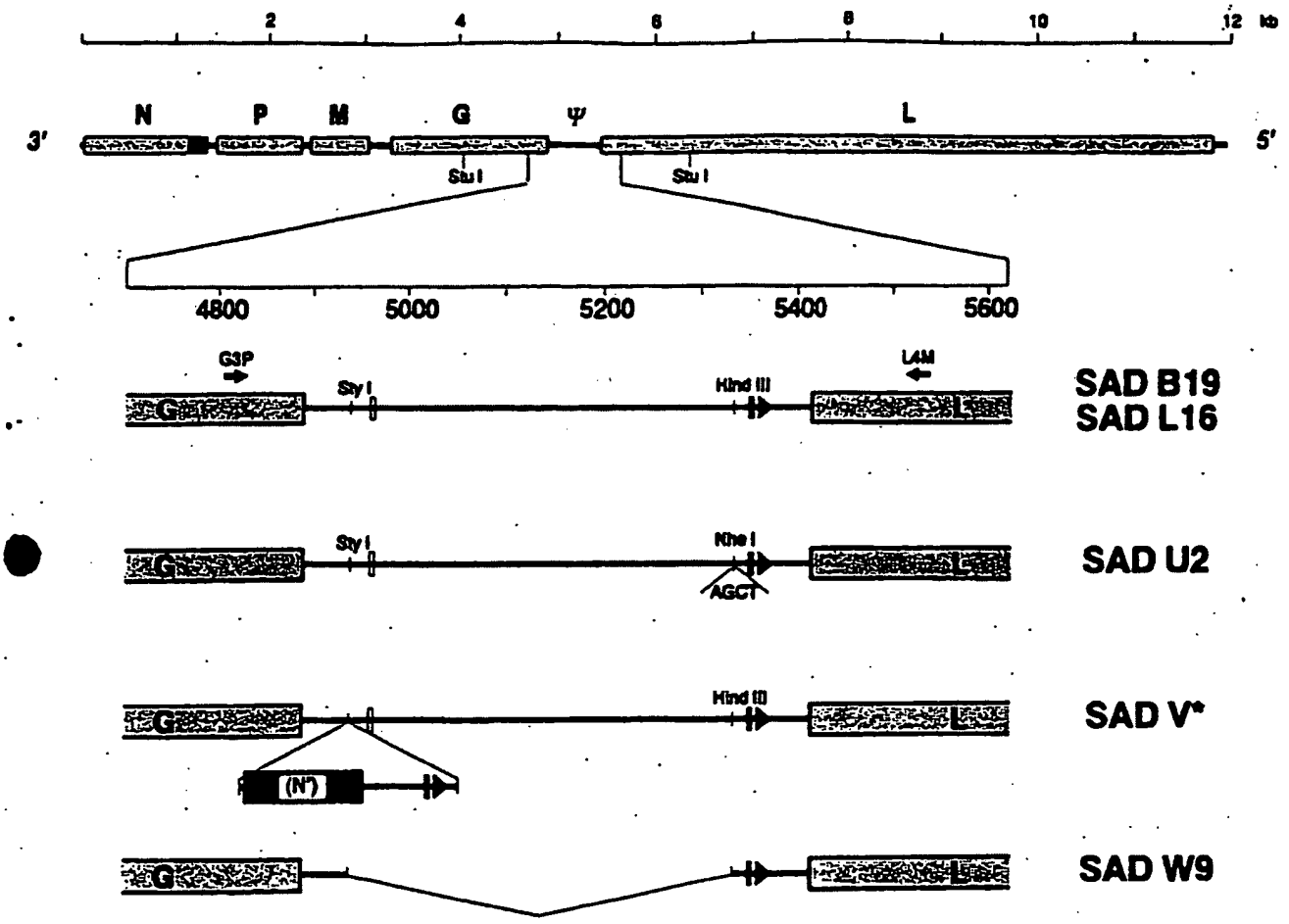
8. A process according to claims 6-7, characterized in that the transcripts of the rabies virus cDNA genome are positive stranded antigenomic RNAs.

9. A process according to claim 6-8, characterized in that the RNA polymerase is T7 RNA polymerase, preferably expressed from a recombinant vaccinia virus.

ABSTRACT

The present invention provides the generation of infectious rabies virus, a nonsegmented negative stranded RNA virus, entirely from cloned cDNA.

This process offers the possibility to introduce mutations into the rabies virus genome by means of recombinant DNA techniques.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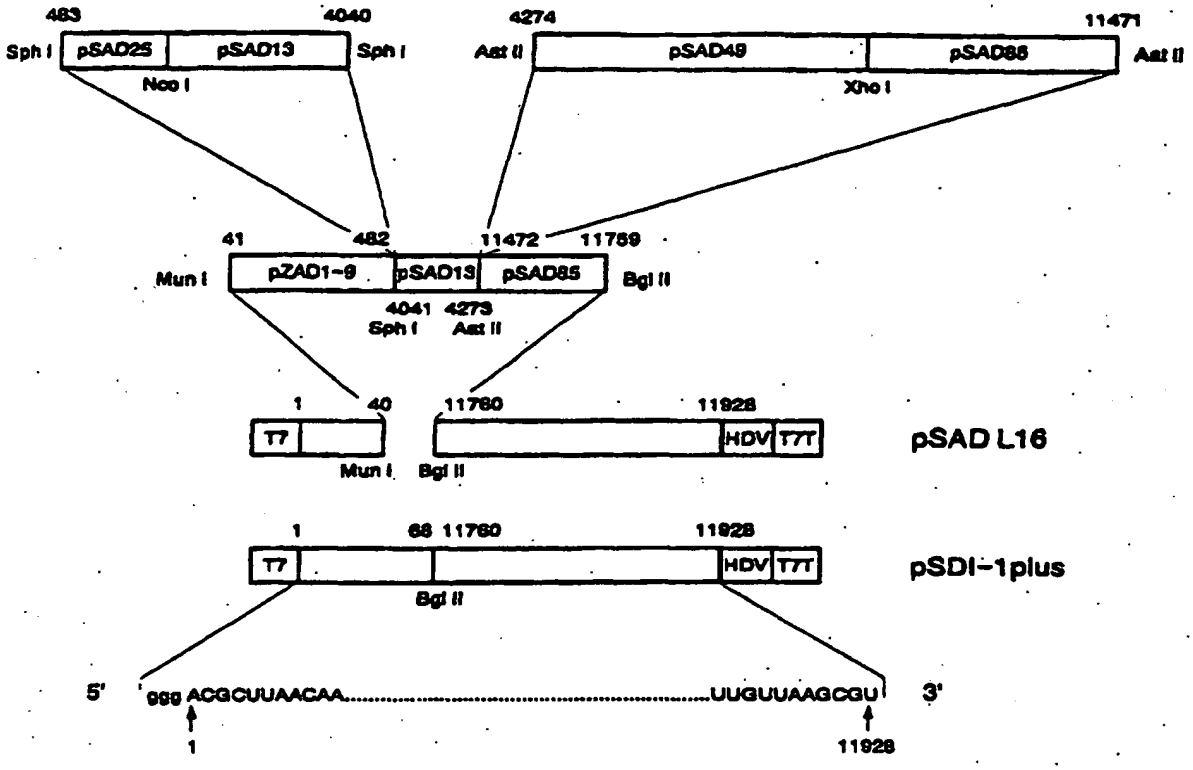
(N')
SAD V*
SAD W9

Figure 1.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11472
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Figure 2.

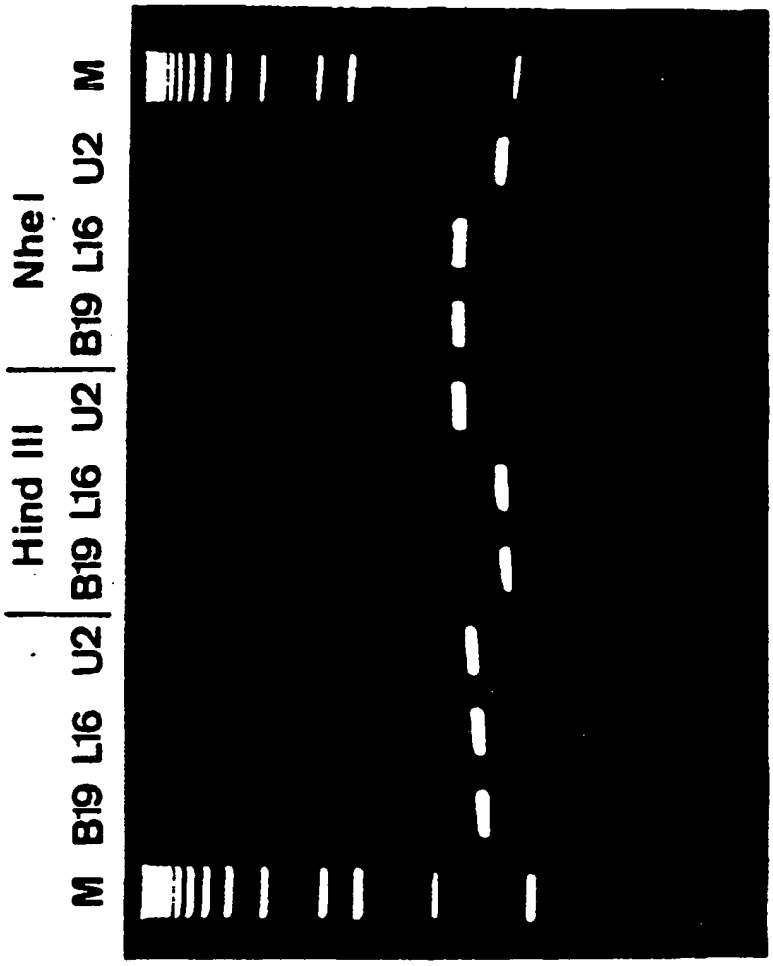

Figure 3.

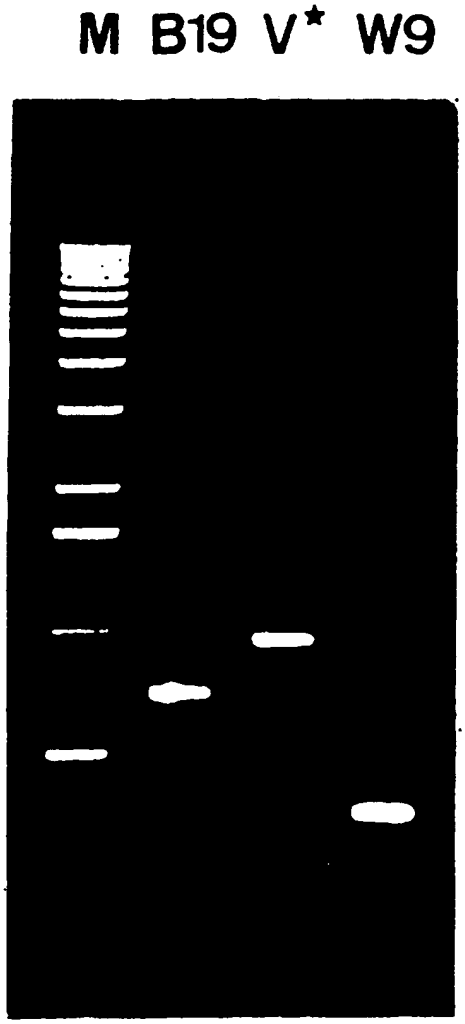

Figure 4.